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(54) Title: TUMOR REJECTION ANTIGENS PRESENTED BY HLA-B44 MOLECULES, AND USES THEREOF

(57) Abstract

Tumor rejection antigens presented by HLA-B44 molecules are described. These peptides are useful in diagnostic and therapeutic methodologies. The tumor rejection antigens are derived from MAGE tumor rejection antigen precursors.

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TUMOR REJECTION ANTIGENS PRESENTED BY HLA-B44 MOLECULES, AND USES THEREOF

RELATED APPLICATION

This application is a continuation-in-part of Serial No. 08/531,864 filed September 21, 1995, which is a continuation-in-part of Serial No. 08/373,636, filed on January 17, 1995, which is in turn a continuation-in-part of copending application Serial No. 08/253,503 filed June 3, 1994 now U.S. Patent No. 5,589,334. All applications are incorporated by reference.

FIELD OF THE INVENTION

This invention relates to isolated peptides, derived from tumor rejection antigen precursors and presented by HLA molecules, HLA-B44 in particular, and uses thereof. In addition, it relates to the ability to identify those individuals diagnosed with conditions characterized by cellular abnormalities whose abnormal cells present complexes of these peptides and HLA molecules, the presented peptides, and the ramifications thereof.

BACKGROUND AND PRIOR ART

The process by which the mammalian immune system recognizes and reacts to foreign or alien materials is a complex one. An important facet of the system is the T cell response. This response requires that T cells recognize and interact with complexes of cell surface molecules, referred to as human leukocyte antigens ("HLA"), or major histocompatibility complexes ("MHCs"), and peptides. The peptides are derived from larger molecules which are processed by the cells which also present the HLA/MHC molecule. See in this regard Male et al., *Advanced Immunology* (J.P. Lipincott Company, 1987), especially chapters 6-10. The interaction of T cell and complexes of HLA/peptide is restricted, requiring a T cell specific for a particular combination of an HLA molecule and a peptide. If a specific T cell is not present, there is no T cell response even if its partner complex is present. Similarly,

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there is no response if the specific complex is absent, but the T cell is present. This mechanism is involved in the immune system's response to foreign materials, in autoimmune pathologies, and in responses to cellular abnormalities.

5   Recently, much work has focused on the mechanisms by which proteins are processed into the HLA binding peptides. See, in this regard, Barinaga, Science 257: 880 (1992); Fremont et al., Science 257: 919 (1992); Matsumura et al., Science 257: 927 (1992); Latron et al., Science 257: 964 (1992).

10   The mechanism by which T cells recognize cellular abnormalities has also been implicated in cancer. For example, in PCT application PCT/US92/04354, filed May 22, 1992, published on November 26, 1992, and incorporated by reference, a family of genes is disclosed, which are

15   processed into peptides which, in turn, are expressed on cell surfaces, which can lead to lysis of the tumor cells by specific CTLs. The genes are said to code for "tumor rejection antigen precursors" or "TRAP" molecules, and the peptides derived therefrom are referred to as "tumor

20   rejection antigens" or "TRAs". See Traversari et al., Immunogenetics 35: 145 (1992); van der Bruggen et al., Science 254: 1643 (1991), for further information on this family of genes. Also see U.S. Patent No. 5,342,774, incorporated by reference.

25   In U.S. Patent No. 5,405,940, the disclosure of which is incorporated by reference, nonapeptides are taught which bind to the HLA-A1 molecule. The reference teaches that given the known specificity of particular peptides for particular HLA molecules, one should expect a particular

30   peptide to bind one HLA molecule, but not others. This is important, because different individuals possess different HLA phenotypes. As a result, while identification of a particular peptide as being a partner for a specific HLA molecule has diagnostic and therapeutic ramifications, these

35   are only relevant for individuals with that particular HLA phenotype. There is a need for further work in the area, because cellular abnormalities are not restricted to one

particular HLA phenotype, and targeted therapy requires some knowledge of the phenotype of the abnormal cells at issue.

The enzyme tyrosinase catalyzes the reaction converting tyrosine to dehydroxyphenylalanine or "DOPA" and appears to be expressed selectively in melanocytes (Muller et al., EMBO J 7: 2715 (1988)). An early report of cDNA for the human enzyme is found in Kwon, U.S. Patent No. 4,898,814. A later report by Bouchard et al., J. Exp. Med. 169: 2029 (1989) presents a slightly different sequence. A great deal of effort has gone into identifying inhibitors for this enzyme, as it has been implicated in pigmentation diseases. Some examples of this literature include Jinbow, WO9116302; Mishima et al., U.S. Patent No. 5,077,059, and Nazzaropor, U.S. Patent No. 4,818,768. The artisan will be familiar with other references which teach similar materials.

U.S. Patent Application 08/081,673, filed June 23, 1993 and incorporated by reference, teaches that tyrosinase may be treated in a manner similar to a foreign antigen or a TRAP molecule - i.e., it was found that in certain cellular abnormalities, such as melanoma, tyrosinase is processed and a peptide derived therefrom forms a complex with HLA molecules on certain abnormal cells. These complexes were found to be recognized by cytolytic T cells ("CTLs"), which then lyse the presenting cells. The ramifications of this surprising and unexpected phenomenon were discussed. Additional peptides have now been found which also act as tumor rejection antigens presented by HLA-A2 molecules. These are described in Serial No. 08/203,054, filed February 28, 1994 and incorporated by reference.

U.S. Patent Application Serial No. 08/233,305 filed April 26, 1994 and incorporated by reference, disclosed that tyrosinase is also processed to an antigen presented by HLA-B44 molecules. The finding was of importance, because not all individuals are HLA-A2⁺. The fact that tyrosinase is processed to an HLA-B44 presented peptide, however, does not provide for a universal approach to diagnosis and treatment of all HLA-B44⁺ tumors, because tyrosinase expression is not

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universal.   Further, the fact that tyrosinase is expressed
by normal cells as well as tumor cells may suggest some
caution in the therapeutic area.

    Population analysis indicates that about 22-24% of the
caucasian population expresses the HLA-B44 molecule. See,
e.g., Imanishi, et al., in Tsuji et al., ed., "HLA 1991,
Vol. I, XIth International Histocompatibility Workshop And
Conference", Oxford University Press, 1991; Lee in Lee, ed.,
The HLA System: A New Approach. Springer, pp. 141-178
(1990).   Prior work has shown that about 76% of all
metastatic melanoma tumors express MAGE-3 (Brasseur, et al.,
Int. J. Cancer 63: 375-380 (1995)). Thus, approximately 17%
of metastatic melanomas in the caucasian population should
present complexes of HLA-B44 and MAGE-3 derived peptides on
their surfaces, with clear implications for diagnosis and
therapy. Five different HLA-B44 alleles have been
identified, as can be seen via Fleischhauer, et al., Tissue
Antigens 37: 133-137 (1991); Petersdorf, et al., Tissue
Antigens 44: 211-216 (1994); Yao, et al., Immunogenetics 40:
310; Yao, et al., Human Immunol 42: 54-60 (1995); Yao, et
al., Immunogenetics 41: 387 (1995). Among the caucasian
population which expresses HLA-B44, 61% present the HLA-
B*4402 allele, and 36% present allele HLA-B*4403. See Yao,
et al., Human Immunol. 42: 54-60 1995). There is a single
difference between these two alleles which occurs at amino
acid 156. In HLA-B*4402, this position is occupied by Asp,
while it is occupied by Leu in HLA-B*4403. Bone marrow
allograft rejections, and Graft versus Host disease have
been observed between donors and recipients which differ
with respect to the HLA-B*4402 and HLA-B*4403 alleles.
Since amino acid 156 is located in the middle of α2 helix
and extends into the peptide binding site, it was of
interest to determine if these different HLA-B44 alleles
present different peptides.

    Khanna, et al., J. Exp. Med. 176: 169-179 (July 1992),
disclose an HLA-B44 binding peptide, which is discussed
further _infra_. The Khanna peptide is not related to the

peptides claimed herein.

Kita, et al., Hepatology 18(5): 1039-1044 (1993), teach a 20 amino acid peptide alleged to bind to HLA-B44 and to provoke lysis.

5   Thorpe, et al., Immunogenetics 40: 303-305 (1994), discuss alignment of two peptides found to bind to HLA-B44, and suggest a binding motif generally. The Thorpe disclosure speaks of a negatively charged amino acid at position 2, and one at position 9 which may be hydrophobic,
10   or positively charged.

Fleischhauer, et al., Tissue Antigens 44: 311-317 (1994) contains a survey of HLA-B44 binding peptides.

It has now been found that the MAGE-3 also expresses a tumor rejection antigen precursor is processed to one tumor
15   rejection antigens presented by HLA-B44 molecules. It is of interest that this peptide differs from a peptide also derived from MAGE-3 and known to bind to HLA-A1, by a single, added amino acid at the N-terminus. This, inter alia, is the subject of the invention disclosure which
20   follows.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows the results of chromium release assays using each of three different cell lines (LB33-MELc1, LB33 EBV-B, and K562), by cytolytic T cell clone 159/5. The data
25   are presented in terms of effector/target ratios vs % of lysis.

Figure 2 shows the result of lysis studies which identified cell variants "A⁻" "B⁻", and "A⁻,B⁻". Again, a chromium release assay was used. Cell line LB33-MELc1 is
30   A⁺B⁺, as is indicated by the positive lysis with both CTL lines tested. CTL 159/93 is anti-A, while CTL 159/5 is anti-B.

Figure 3 shows results obtained when the variant A⁻B⁻ was transfected with coding sequences for each of HLA-A28,
35   HLA-B44, and HLA-Cw7, as compared to a control line. The results are depicted in terms of the sensitive TNF release assay (pg/ml), where CTL 159/5 was used.

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Figure 4 shows TNF release by CTL 159/5, where COS cells were transfected with HLA-B44, or HLA-B44 plus a nucleic acid molecule in accordance with this invention.

Figure 5A depicts ^{51}Cr release in EBV-B cells, when
5   contacted with CTL 159/5.

Figure 5B is similar, but uses LB33-MEL B⁻ cells. In each of figures 5A and 5B, the antigenic peptide of the invention was contacted to the cells prior to contact with the CTLs.

10   Figure 6 shows the lytic activity of various autologous CTL clones on antigen loss variants derived from melanoma clonal line LB33.MEL.A-1.

Figure 7 presents results showing expression of HLA-A24, A28 and B13 molecules by antigen loss variants of LB33-
15   MEL.A-1. Tumor cells had been incubated with mouse antibodies against particular HLA molecules, and were then labeled with fluorescein tagged goat anti-mouse antibodies.

Figure 8 shows the production of tumor necrosis factor (TNF) by CTL clones stimulated by antigen loss variants,
20   transfected with various HLA alleles. Untransfected LB33-MEL.A-1 cells were used as controls, as were antigen loss variants. The CTL clones used were 159/3, 159/5 and 204/26, 179 c/50, and 202/1 corresponding to anti-A, anti-B, an anti-Ca anti-Cb, and anti-D CTLs, respectively.

25   Figure 9 sets forth further data regarding the cytolytic activity of the CTL clones described in figures 6 and 8. The cell line LB33-MEL.A had been obtained following surgery in 1988. Cell line LB33-MEL.B was obtained from a metastasis which developed in the patient in 1993.

30   Figure 10A depicts the lytic activity of anti-E CTL clone LB33-CTL-269/1 on autologous melanoma cells, while figure 10B shows production of TNF by the same CTL clone, following stimulation by LB33-MEL.B-1 cells. The stimulator cells (10,000/microwell) had been incubated for 16 hours
35   with 3000 CTLs. The concentration of TNF released by the CTLs had been measured using TNF sensitive WEHI-164c13 cells. Anti HLA-A24 monoclonal antibody C7709A2 was used to

inhibit CTL stimulation, by adding a 1/100 dilution of ascites fluid obtained from mice inoculated with the hybridoma cells.

Figures 11A and 11B show the results of assays, wherein SEQ ID NOS: 17, 18, 19 and 3 were tested in competitive binding assays.

Figure 12 depicts the result obtained when SEQ ID NO: 17 was used in connection with cells which naturally present HLA-B44, in ^{51}Cr release assays.

Figure 13 summarizes the results of ^{51}Cr lysis assays where the target cells were naturally HLA-B44 positive, such as cancer cell lines.

Figure 14 shows the results of studies wherein SEQ ID NOS: 17 and 3 were tested in competitive binding assays.

Figure 15A shows the result of lytic assays, in a ^{51}Cr release assay, where lymphocytes obtained from donor LB816 were tested against HLA-B*4402 presenting cells. Figure 15B presents parallel information, using lymphocytes obtained from donor LB822 CTL LB822, with HLA-B*4403 presenting cells.

Figure 16 presents data which confirm the specificity of CTLs specific for complexes of SEQ ID NO: 17 and HLA-B44 molecules.

Figure 17 presents data which confirm the specificity of CTLs specific for MAGE-3 and HLA-B44.

Figure 18 summarizes results obtained when cells which naturally present HLA-B44 molecules and also express MAGE-3 were tested with the CTLs discussed in this application.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

Example 1

Melanoma cell line LB33-MEL which has been available to researchers for many years, was used in the following experiments. A clone derived therefrom was also used. The clone is referred to hereafter as LB33-MELc1.

Samples containing mononuclear blood cells were taken from patient LB33. The melanoma cell line was contacted to the mononuclear blood cell containing samples. The mixtures

were observed for lysis of the melanoma cell lines, this lysis indicating that cytolytic T cells ("CTLs") specific for a complex of peptide and HLA molecule presented by the melanoma cells were present in the sample.

The lysis assay employed was a chromium release assay following Herin et al., Int. J. Cancer 39:390-396 (1987), the disclosure of which is incorporated by reference. The assay, however, is described herein. The target melanoma cells were grown _in vitro_, and then resuspended at 10^7 cells/ml in DMEM, supplemented with 10 mM HEPES and 30% FCS (i.e., from fetal calf serum) and incubated for 45 minutes at 37°C with 200 μCi/ml of Na(^{51}Cr)O$_4$. Labelled cells were washed three times with DMEM, supplemented with 10 mM Hepes. These were then resuspended in DMEM supplemented with 10 mM Hepes and 10% FCS, after which 100 ul aliquots containing 10^3 cells, were distributed into 96 well microplates. Samples of PBLs were added in 100 ul of the same medium, and assays were carried out in duplicate. Plates were centrifuged for 4 minutes at 100g, and incubated for four hours at 37°C in a 5.5% CO_2 atmosphere.

Plates were centrifuged again, and 100 ul aliquots of supernatant were collected and counted. Percentage of ^{51}Cr release was calculated as follows:

$$\% \ {}^{51}Cr \ release = \frac{(ER-SR)}{(MR-SR)} \times 100$$

where ER is observed, experimental ^{51}Cr release, SR is spontaneous release measured by incubating 10^3 labeled cells in 200 ul of medium alone, and MR is maximum release, obtained by adding 100 ul 0.3% Triton X-100 to target cells.

Those mononuclear blood samples which showed high CTL activity were expanded and cloned via limiting dilution, and were screened again, using the same methodology.

The same method was used to test target K562 cells. When EBV-B cells were used, the only change was the replacement of DMEM medium by Hank's medium, supplemented with 5% FCS.

These experiments led to isolation of CTL clones LB33-CTL-159/5. Figure 1 shows that this clone lysed tumor cells, but not EBV-B cells, or K562 cells.

Following the same protocol, a second CTL clone, i.e., LB33-CTL-159/3 was isolated. These lines will be referred to as "159/5" and "159/3", respectively. This second CTL has specificity differing from 159/5. This was ascertained following isolation of two antigen loss variants which (i) are lysed by 159/5 but not 159/3 and (ii) are not lysed by 159/5 and are lysed by 159/3. These variants are referred to as A⁻ and B⁻, respectively.

The A⁻ variant was then immunoselected with 159/5, and a third variant was obtained, which was not lysed by either 159/5 or 159/3. This variant is referred to as A⁻B⁻. Figure 2 summarizes the results of the lysis assays, leading to isolation of the variants.

Example 2

It was of interest to determine the pattern of HLA expression of variant A⁻B⁻. The patient from whom parent line LB33-MEL was derived was typed as HLA-A24, A28, B13, B44, Cw6, Cw7. When PCR expression analysis was carried out, it was found that both LB33-MELc1, and the B⁻ variant express all six alleles; however, the A-B⁻ variant does not express HLA-A28, B44, and Cw7. As a result, it was concluded that one of these HLA molecules presents the antigen leading to lysis by CTLs. The following example explores this further.

Example 3

Samples of the A⁻B⁻ variant were transfected by plasmid pcDNA-I/AmpI which had cloned therein, one of HLA-A28, HLA-

B44, or HLA-Cw7. Following selection, the cells were tested in a TNF release assay, following Traversari, et al., Immunogenetics 35: 145-152 (1992), incorporated by reference herein. The results are summarized in figure 3, which shows that HLA-B44 is clearly implicated in the presentation of the antigen.

Example 4

Once the presenting HLA molecule was identified, studies were carried out to identify the molecule, referred to hereafter as the "tumor rejection antigen precursor" or "TRAP" molecule which was the source of the presented peptide.

To do this, total mRNA was isolated from cell line LB33-MELc1. The messenger RNA was isolated using an oligo-dT binding kit, following well recognized techniques. Once the messenger RNA was secured, it was transcribed into cDNA, again using standard methodologies. The cDNA was then ligated to EcoRI adaptors and cloned into the EcoRI site of plasmid pcDNA-I/Amp, in accordance with manufacturer's instructions. The recombinant plasmids were then electrophorated into DH5α E. coli (electroporation conditions: 1 pulse at 25 μfarads, 2500 V).

The transfected bacteria were selected with ampicillin (50 μg/ml), and then divided into pools of 100 bacteria each. Each pool represented about 50 different cDNAs, as analysis showed that about 50% of plasmids contained an insert. Each pool was amplified to saturation, and plasmid DNA was isolated via alkaline lysis, potassium acetate precipitation and phenol extraction, following Maniatis et al., in Molecular Cloning: A Laboratory Manual (Cold Spring Harbor, N.Y., 1982). Cesium gradient centrifugation was not used.

The amplified plasmids were then transfected into eukaryotic cells. Samples of COS-7 cells were seeded, at 15,000 cells/well into tissue culture flat bottom microwells, in Dulbeco's modified Eagles Medium ("DMEM") supplemented with 10% fetal calf serum. The cells were

incubated overnight at 37°C, medium was removed and then replaced by 30 μl/well of DMEM medium containing 10% Nu serum, 400 μg/ml DEAE-dextran, 100 μM chloroquine, and 100 ng of a plasmid containing cDNA for HLA-B44 from LB33. Following four hours of incubation at 37°C, the medium was removed, and replaced by 50 μl of PBS containing 10% DMSO. This medium was removed after two minutes and replaced by 200 μl of DMEM supplemented with 10% of FCS.

Following this change in medium, COS cells were incubated for 48 hours at 37°C. Medium was then discarded, and 2000 cells of 159/5 were added, in 100 μl of Iscove's medium containing 10% pooled human serum and 25 U/ml IL-2. Supernatant was removed after 24 hours, and TNF content was determined in an assay on WEHI cells, as described by Traversari et al., Immunogenetics 35: 145-152 (1992), the disclosure of which is incorporated by reference. One pool stimulated TNF release above background, and these bacteria were cloned, and used in the following experiment.

Example 5

Plasmid DNA was extracted from the bacteria cloned in Example 4, transfected into a new sample of COS cells in the same manner as described supra, and the cells were again tested for stimulation of 159/5. A positive clone was found in clone 350/2, as demonstrated by data summarized in figure 4A.

In order to confirm the results obtained to this point, the human choriocarcinoma cell line JAR, which is readily available from the American Type Culture Collection, was used. This cell line does not express HLA molecules, nor is it recognized by CTL 159/5. When JAR was transfected with HLA-B44 cDNA, it was still not recognized by CTL 159/5. Co-transfection with HLA-B44 and 350/2 cDNAs, however, led to lysis, as is seen in figure 4B.

The plasmid from the positive clone was removed, and sequenced following art known techniques. Information shows that the plasmid insert was 1896 base pairs long, and showed no homology with any sequences in data banks. The

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nucleotide sequence is set forth herein as SEQ ID NO: 1.

Example 6

In order to ascertain the peptide which was the tumor rejection antigen, fragments of SEQ ID NO: 1, averaging about 300 base pairs, were amplified via PCR, cloned into pcDNA-1/Amp, and then cotransfected into COS cells with plasmid encoding HLA-B44, following the protocols of the preceding examples. These experiments led to identifying the region corresponding to amino acid residues 683-955 of SEQ ID NO: 1 as encoding the antigenic peptide. This region was compared to the peptide described by Khanna, et al., J. Exp. Med. 176: 169-176 (7/92), and the peptide described in Serial No. 08/233,305, filed April 26, 1994, i.e.:

Glu Glu Lys Leu Ile Val Val Leu Phe (SEQ ID NO: 2) corresponds to these residues. As such, a peptide corresponding to this sequence was synthesized, and used to sensitize HLA-B44$^+$ cell lines. The results are shown in figures 6A and 6B, which depict the results of a ^{51}Cr release assay using EBV transformed B cells (figure 6A), and the B$^-$ variant described supra (figure 6B). The cells were incubated with varying concentrations of the peptide for 30 minutes at 37°C, before adding CTL 159/5 (effector/target ratio: 10:1). Half maximal lysis was obtained with 100-200 ng/ml of peptide.

Example 7

Examples 1-6, set forth supra, describe work using the cell line LB33-Melcl. Additional cell lines were also derived from a cutaneous metastasis from patient LB33. One such line is LB33-MEL.A-1, which is used in the example which follows.

First, the cell line was used, in the same manner that the cell line of examples 1-6 was used (Herin et al., supra). Blood mononuclear cells (10^6/well), were stimulated with irradiated tumor cells (3/10^5 cells/well), in 2 ml of Iscove's medium, supplemented with 10% pooled human serum, asparagine-glutamine-arginine (36 mg/ml, 216 mg/ml, 116 mg/ml, respectively), 2-mercaptoethanol (0.05 mM), and 5

U/ml of human IL-4.   IL-2 (10 U/ml) was added on the third
day of cultivation.   Sensitivity of the tumor cells to
autologous CTLs was determined as in example 1, supra.   The
experiment yielded 82 stable cytolytic T lymphocytes,

5   derived from seven independent cultures.   All of these CTLs
were CD8$^+$.   They were specific for tumor cells in that they
lysed LB33-MEL.A-1 cells, but not K562, or autologous, EBV
transformed cells.

Example 8

10   The fact that LB33-MEL.A-1 cells were lysed by
autologous CTLs suggested the next experiment, which was to
identify the antigens recognized by establishing antigen
loss variants.

To do this, samples of the cell line were selected,

15   four times, with the autologous CTL clone LB33-CTL 159/3,
described supra.   Each round of selection involved
incubating, for 2-6 hours, $2-3\times10^7$ adherent tumor cells with
a similar number of CTLs, in the same manner described
supra.   In each round, CTLs were washed away following the

20   incubation, and the surviving adherent tumor cells were
amplified prior to the next round of selection.

This procedure resulted in a clone resistant to CTL
159/3; however, when tested with additional autologous CTLs,
it was found that CTL 159/5, described supra, did lyse the

25   loss variant, as did additional CTL clones, including
204/26, and 202/1.   Please see figure 6, the column labelled
"MEL.A-1.1".   Similarly, additional cell lines were
established which were not lysed by one of these four CTL
clones, but was lysed by the others.   Note figure 6.   Thus,

30   at least four different antigens were found to be presented
on the surface of LB33-MEL.A-1, because four distinct
antigen-loss variants were identified.   As set forth in
figure 6, then, LB33-MEL.A-1 is considered "A$^+$B$^+$C$^+$D$^+$" for
antigen expression (lysed by all of CTL 159/3, 159/5,

35   204/26, and 202/1); MEL.A-1.1 is A$^-$B$^+$C$^+$D$^+$ (not lysed by
159/3, lysed by others); MEL.A-1.2 is A$^+$B$^-$C$^+$D$^+$ (not lysed by
159/5; lysed by others), MEL.A-1.3 is A$^+$B$^+$C$^-$D$^+$ (not lysed by

204/26; lysed by others), and MEL.A-1.4 is $A^-B^+C^+D^-$ (not lysed by 202/1 or 159/3). Further, cell line MEL.A-1.1.1 was isolated, which was $A^-B^-C^+D^-$ (lysed only by 204/26).

5   When the 82 CTLs identified via example 7 were tested on these lines, 29 anti-A, 29 anti-B, 10 anti-C, and 14 anti-D clones were identified, suggesting that there were no other antigens being presented.

       Selection with anti-D CTL clone 202/1 led to identification of a line which was also resistant to the
10   anti-A CTL clone (159/3), as did selection with anti-B CTL (i.e., the resulting $A^-B^-C^+D^-$ line). This result suggests that A^-D^- and $A^-B^-D^-$ antigen loss variants were actually HLA loss variants, with antigens A, B and D sharing the same HLA presenting molecule, or that different class I molecules had
15   been lost together with the antigen loss variants. The following experiments pursued this issue.

Example 9

       The patient from whom the LB33 cell lines had been developed had been serologically typed, previously, as HLA-
20   A24, A28, B13, B44, Cw6, Cw7. Studies were then carried out to determine the expression of HLA class I genes by the cell lines.

       Semi-quantitative conditions for DNA amplification by PCR were established in order to assess the expression of
25   each of the six class I alleles by the different LB33-MEL tumor cell clones. The Amplification Refractory Mutation System (ARMS) PCR methodology proposed by Browning et al, that relies on the perfect nucleotide matched needed at the 3' end of primers to ensure specificity of DNA
30   amplifications was used. See Browning et al, Proc. Natl. Acad. Sci. USA 90: 2842 (1993) incorporated by reference herein. On the basis of sequences obtained in typing LB33, allele-specific primers that enabled discrimination of each one of the six alleles from the five others (5' primer
35   followed by 3' primer) were synthesized.

for A24: 5'-GCCGGAGTATTGGGACGA and 5'-GGCCGCCTCCCACTTGC (SEQ
ID NO: 5 and 6)

for A28: 5'-GGAGTATTGGGACCGGAAG and 5'-GGCCGCCTCCCACTTGT
(SEQ ID NO: 7 and 8)

for B13: 5'-CGCCACGAGTCCGAGGAT and 5'-CCTTGCCGTCGTAGGCTA
(SEQ ID NO: 9 and 10)

for B44: 5'-CGCCACGAGTCCGAGGAA and 5'-CCTTGCCGTCGTAGGCGT
(SEQ ID NO: 11 and 12)

for Cw6: 5'-CCGAGTGAACCTGCGGAAA and 5'-GGTCGCAGCCATACATCCA
(SEQ ID NO: 13 and 14)

for Cw7: 5'-TACAAGCGCCAGGCACAGG and 5'-CTCCAGGTAGGCTCTGTC
(SEQ ID NO: 15 and 16)

To carry out semi-quantitative measurements of expression,
27 cycles of PCR amplification of reverse transcribed RNA
were carried out with each set of primers and DNA
amplification was found to be in the linear range observed.
The quantity of the amplified DNA was visually assessed with
agarose gels stained with ethidium bromide. These
quantities were compared to those obtained with a standard
curve containing the products of RT-PCR amplification of
serial dilutions of RNA from LB33-MEL.A-1 cells. The
expression of samples was normalized for RNA integrity by
taking into account the expression level of the ß-actin
gene. The results were expressed relative to the level of
expression by LB33-MEL.A-1 cells. The results of this work
are set forth in Table 1, which follows. A "+++" indicates
expression corresponding to more than half that of the LB33-
MEL.A-1 cells, "++" means that expression was between 1/8
and 1/2 of that of LB33-MEL.A-1, a "+" means that
expression was less than 1/8 of that of LB33-MEL.A-1
expressed and "-" means there was no expression.

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TABLE 1.
Expression of HLA class I by the antigen-loss variants
derived from LB33-MEL.A-1 cells.

Expression of	LB33-MEL.A-1	LB33-MEL.A tumor cells Antigen-loss variants				
		A⁻	B⁻	C⁻	A⁻D⁻	A⁻B⁻D⁻
A.	Gene Expression					
A24	+++	+++	+++	−	++	+++
A28	+++	+++	+++	+++	+	−
B13	+++	+++	+++	+	+++	+++
B44	+++	+++	+++	+++	++	−
Cw6	+++	+++	+++	+	+++	+++
Cw7	+++	++	+++	+++	+	−

As seen, both MEL.A-1 cells, and B⁻ variant expressed
similar levels of all six HLA alleles. The A⁻ variant
showed an approximately 4-fold decrease in expression of
Cw7. The remaining antigen loss variants showed decreases
in expression of sets of three alleles. For C⁻ cells,
reduced levels of expression for HLA-A24, B13, and Cw6 were
found, while A⁻D⁻, and A⁻B⁻D⁻ variants showed reduction in
A28, B44, and Cw7 expression. This suggests that A24-B13-
Cw6, and A28-B44-Cw7 constitute two HLA class I haplotypes
of patient LB33, and that reduced expression of these
haplotype probably accounted for loss of antigen expression
by the immunoselected tumor cells.

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Example 10

The next experiments were designed to confirm a correlation between HLA gene expression, and lysis by CTLs. To do this, the expression of a given HLA gene, as determined supra, was compared with the results obtained using a standard antibody assay. Only A24, A28 and B13 were tested, using murine antibodies specific thereto (C7709A1 for A24; 2.28M1 for A28, and TÜ 48 for B13). Binding of antibody was determined by incubation with antibody, washing and then contacting with goat anti-mouse Ig antibodies, coupled to fluorescein. The cells were then analyzed by flow cytometry, a standard technique.

Table 2 summarizes the results, which are also shown in figure 7. In table 2 that follows, the indicated level of HLA expression corresponds to the mean intensity of fluorescence shown in figure 6. Values are expressed relative to levels found in LB33-MEL.A-1 cells.

It appears from these results that when levels of HLA expression estimated to range below 1/8 of that of LB33-MEL.A-1 cells, undetectable or barely detectable levels of HLA surface molecules are found, thus suggesting that antigen presentation to CTL was unlikely for the given HLA molecule.

In view of this, and assuming that C^-, A^-D^- and $A^-B^-D^-$ selected cells had lost expression of antigen because of lack of HLA molecules, it appeared to be the case that the class I presenting molecules for antigen A were A28 or Cw7, B44 for antigen B, A24 or B13 or Cw6 for antigen C, and A28 or Cw7 for antigen D.

Table 2

LB33-MEL.A-1				Antigen-loss variants	

Expression of	A⁻	B⁻	C⁻	A⁻D⁻	A⁻B⁻D⁻

Expression of surface antigen

	A⁻	B⁻	C⁻	A⁻D⁻	A⁻B⁻D⁻
A24	100	33	13	4	41	95
A28	100	29	14	3	1	1
B13	100	27	22	1	40	230

Example 11

The experiments detailed above were followed by
additional work to determine, definitively, the presenting
molecules for the antigens expressed by the LB33-MEL.A cells.
To do this, tumor cells which had lost expression of
particular HLA class I molecules were transfected, using the
classic calcium phosphate precipitation method, with
expression vector pcDNA3, into which the particular class I
cDNA was cloned. This vector contains the neo^R marker.
Transfectants were selected with 1.5 mg/ml of G418, and were
then used to stimulate CTL clones, using the TNF assay set
forth in the previous examples.

Figure 8 depicts these results. Expression of antigen B
was restored in A⁻B⁻D⁻ cells by transfection with a plasmid
carrying HLA-B44, but not with plasmids containing HLA-A28 or
HLA-Cw7. The expression of antigen C was restored in C⁻ cells
by transfection with HLA B13. Four other anti-C CTL clones
also recognized C⁻ cells, but five other anti-C CTL clones,
including depicted CTL 179C/50, did not; rather, these CTLs
recognized C⁻ cells transfected with HLA-Cw6. Thus, it may be
concluded that there are _two_ groups of anti-C CTL clones. One

recognizes an antigen presented by HLA-B13, and the other an antigen presented by HLA-Cw6. As for antigen D, A^-D^- cells were restored to A^-D^+ via transfection with HLA-A28. None of the cDNA restored expression of antigen A (i.e., tested HLA A28, B44, Cw7), although it clearly is presented by HLA-class I molecules, because lysis by anti-A CTLs is completely inhibited by anti-class I monoclonal antibody W6/32. It is possible that this antigen may be presented by a non-A, B, C class I molecule, of which two alleles were present in patient LB33, one of these being lost, together with the A28-B44-Cw7 haplotype in A^-D^-, $A^-B^-D^-$ cells.

The results for antigen C have led to a change in nomenclature. There are two antigens referred to as antigen, Ca and antigen Cb, hereafter.

Example 12

In further experiments, the question of whether or not cells of the line LB33-MEL.B could be recognized by autologous cell lines, was addressed.

Irradiated LB33-MEL.B.1 cells were used in the same manner as was used, supra (Herin, et al), to stimulate autologous lymphocytes. The lymphocytes had been taken from patient LB33 in 1990 or 1994.

Only the lymphocytes from 1994 lysed LB33-MEL.B-1 cells; however, they did not lyse LB33-MEL.A cells. Thus, the LB33-MEL.B-1 line presents an antigen not found on LB33-MEL.A.

The experiments described herein parallel those described supra and, as in the prior experiments, another panel of $CD8^+$ CTL clones were established. The panel of reactivity of CTL 269/1 is shown in figure 10A. Note reaction with "MEL.B-1", but not "MEL.A-1". The new antigen defined thereby is referred to as LB33-E.

In antibody inhibitory experiments, mAbs to HLA-A24 inhibited lysis. This is shown in figure 10B. Hence, the "E" antigen is presented by HLA-A24.

Example 13

Fleischhauer et al., Tissue Antigens 44: 311-317 (1994), incorporated by reference, teach a consensus motif for HLA-B44

20

binding. This motif is described as a nine or ten amino acid
polypeptide, where Glu predominates at second position, Tyr or
Phe is present at the last position (position 9 or 10), and
hydrophobic residues, such as Met, are at the third position.

5   The MAGE-3 TRAP amino acid sequence contains a stretch of
amino acids at position 167-176, which corresponds to this
motif. The amino acid sequence is:

        Met Glu Val Asp Pro Ile Gly His Leu Tyr
(SEQ ID NO: 17).

10   The HLA-B44 motif is known to contain at least two major
subtypes, referred to as HLA-B* 4402 and HLA-B* 4403. The MHC
molecule appears on 23% of all caucasians. When this figure
is combined with standard analyses of melanoma, it is
concluded that 15% of caucasian melanoma patients should

15   present HLA-B44 on the surface of their melanoma cells. Thus,
it is of great interest to determine if the peptide of SEQ ID
NO: 17 or related molecules can in fact be used to identify
HLA-B44 cells, and to provoke their lysis following binding to
the MHC molecule. As noted in prior examples, the peptide of

20   SEQ ID NO: 2 was shown to bind to HLA-B44 positive cells. A
peptide was designed which was similar to SEQ ID NO: 2, except
for having Ala at position 8, rather than Leu. This new
peptide, i.e.:

        Glu Glu Lys Leu Ile Val Val Ala Phe
25   (SEQ ID NO: 18), was tested in a competition assay with SEQ ID
NO: 2. This peptide was used in view of result obtained in
experiments not reported here. Briefly, derivatives of SEQ ID
NO: 2 were prepared, wherein each derivative contained an Ala
at a position not occupied by Ala in SEQ ID NO: 2. CTL clone

30   159/5 was slightly better at recognizing complexes containing
SEQ ID NO: 18 than SEQ ID NO: 2, making it an excellent
reagent for competitive assays. Competition was carried out
using C1R cells, described by Storkus et al., J. Immunol
138:1657-1659 (1987). These C1R cells are MHC class I

35   negative, lymphoblastoid cells. The C1R cells were
transfected with a cDNA for HLA-B*4402, or with a genomic DNA
for HLA-B*4403, using the same methodology given supra. The

21

cDNA for HLA-B*4402 is set forth by Fleischhauer, et al,
Tissue Antigens 44: 311-317 (1994), while the genomic DNA for
HLA-B*4403 is given by Fleischhauer, et al. (1990) New Eng. J.
Med 323:1818-1822 (1990). Both papers are incorporated by
5   reference.

The cells were labelled with ^{51}Cr for one hour at 37°C, in
the presence of anti-HLA class I monoclonal antibody W6/32
(30% (v/v) of culture medium of the hybridoma cells). This
increases the ability of the cells to present antigenic
10   peptides to T cells.

Labelled cells were washed, and incubated for 30 minutes
at 20°C, in serum free medium, together with various
concentrations of competitor peptides. These peptides
included:

15   Ser Glu Ile Trp Arg Asp Ile Asp Phe
(SEQ ID NO: 3)
which binds to HLA-B44 molecules, as discussed, supra
                 Phe Leu Arg Gly Arg Ala Tyr Gly Leu
(SEQ ID NO: 19),
20   which is encoded by EBV gene EBNA-3A and binds to HLA-B8
(Burrows, J. Exp Med 171:345-349 (1990)), and SEQ ID NO: 17.

The peptide of SEQ ID NO: 18 was then added in the serum
free culture medium at a final concentration of 45 ng/ml,
(C1R-B4402$^+$ cells), or 160 ng/ml (C1R-B4403$^+$ cells). The
25   cells were incubated for 30 minutes at 20°C, and washed twice
in Iscove's medium plus 2% fetal calf serum. The CTL clone
LB33-CTL 159/5 was added in Iscove's medium and 10% human
serum, at an E:T ratio of 20. The release of ^{51}Cr was measured
after three hours, and is shown in figures 11A and 11B, for
30   C1R-B*4402 and C1R-B*4403 cells. The data presented in figure
11, show clear evidence of competition.

Example 14

Additional experiments were then carried out following
those described in Example 13.
35   Cytolytic T cell clones (CTLs) were derived from two
subjects, referred to as LB 816 and LB 822, respectively.
These subjects showed no evidence of cancer.

Blood mononuclear cells (BMCs) were isolated from the subjects, using density gradient centrifugation. T lymphocytes in the BMCs were purified by rosetting, using sheep red blood cells which had been treated with

5 aminoethylisothiouronium bromide, and then labelled with an anti-CD8 monoclonal antibody coupled to magnetic microbeads. The CD8⁺ cells were sorted by passage through a magnetized area, and then stored at -80°C in Iscove's culture medium, supplemented with 10% human serum, 116 mg/l L-arginine, 36

10 mg/ml L-asparagine, and 216 mg/l of L-glutamine, 0.05mM 2-mercaptoethanol, and 10% DMSO.

Any non-rosetting BMC were left to adhere for two hours at 37°C on tissue culture plates. Non-adherent cells were discarded, and adherent cells cultured for seven days in the

15 presence of IL-4 (50 U/ml), and GM-CSF (100 ng/ml). The resulting population was enriched for antigen presenting cells ("APCs"; in this case, dendritic cells or macrophages). Then, from 5×10^5 to 10^6 of these cells were incubated in 2 ml wells for four hours, at 37°C, in 400 ul Iscove's medium

20 supplemented with 2.5 ug/ml of human ß2 microglobulin, and 50 ug/ml of the peptide of SEQ ID NO: 17. Adherent, peptide pulsed cells were then irradiated at 5000 rads, and washed. Next, 2×10^6 autologous CD8⁺ T cells were added, in culture medium, supplemented with 1000 U/ml of IL-6, and 5 ng/ml of

25 IL-12.

Seven days later, lymphocytes were restimulated with adherent, autologous BMCs, pulsed with peptide as above. 5×10^6 BMCs were left to adhere for two hours at 37°C, in 400 ul Iscove's medium containing ß2-microglobulin and SEQ ID NO:

30 17, as discussed above. Any peptide pulsed, adherent cells, were irradiated and washed. Responder cells were then added, in culture medium supplemented with 10 U/ml of IL-2, and 5 ng/ml of IL-7.

On day 14, the lymphocytes were restimulated with

35 autologous BMCs pulsed with SEQ ID NO: 17. The BMCs were incubated, at 2×10^7 cells/ml, in the Iscove's medium containing B_2-microglobulin and SEQ ID NO: 17. After two

hours of incubation (20°C), peptide pulsed BMCs were
irradiated, washed, and resuspended at $2x10^6$ cells/ml in
culture medium augmented with IL-2 and IL-7, as above.
Samples of these stimulator cells ($2x10^6$), were added to each
5 well which contained responder cells.

The responder lymphocytes were cloned on day 21.
Anywhere from 10 to 0.3 cells/well were seeded in microwells,
in culture medium which had been supplemented with 50 U/ml of
IL-2, and 5 U/ml of IL-4. These were then stimulated by
10 adding allogenic EBV transformed B cells (LG2-EBV-B) and
irradiated at 10,000 rads, at 20,000 cells per well, and one
of (i) peptide pulsed HLA-B4402⁺ cells, or (ii) peptide pulsed
HLA-B4403⁺ cells. For (i) or (ii), irradiation was at 15,000
rads, at 8000 cells per well.
15 Microcultures were restimulated every week in the same
way they were on the 21st day. The one change was that at
days 28 and 35, 40,000 and 60,000 EBV-B cells respectively
were added per well, as compared to 20,000 at day 21.

Between days 41 and 52, aliquots of the proliferating
20 microcultures were transferred into V-bottom microwells, in
order to test for lytic activity against HLA-B4402⁺ or HLA-
B4403⁺ target cells, pulsed and not pulsed with SEQ ID NO: 17.

Any microcultures which showed anti-peptide lytic
activity were restimulated with $5x10^4$ irradiated, peptide-
25 pulsed B4402⁺ or B4403⁺ cells, plus $5x10^5$ irradiated LG2-EBV-B
cells, in 800 ul of culture medium augmented with 50 U/ml of
IL-2, and 5 U/ml of IL-4.

After seven days, the CTL clones were restimulated every
week with $2 X 10^5$ irradiated peptide pulsed B4402⁺ or B4403⁺
30 cells, together with 10^6 irradiated LG2-EBV-B cells, as
described supra. In this way, CTLs LB 816-CTL-340 A/1, and
LB822-CTL-346A/1 were obtained. These CTLs are specific for
complexes of SEQ ID NO: 17 and either HLA-B*4402, or HLA-
B4403, respectively.
35 **Example 15**

In a further set of experiments, HLA-B4402⁺ or HLA-B4403⁺
EBV-transformed B cells which do not express MAGE-3 were

labelled with ^{51}Cr in the presence of monoclonal antibody W6/32, for 1 hour, at 37°C, Brodsky, et al, J. Immunol 128:129-135 (1982). The cells were washed, and incubated for 30 minutes at 20°C in serum free medium, using varied

5 concentrations of SEQ ID NO: 17. Each CTL described in Example 14 was tested in a ^{51}Cr release assay, also as described, with chromium release being measured after four hours.

The results, set forth in figure 12, shows that the

10 peptide did, in fact, provoke lysis.

Example 16

In the following experiments, additional tumor cell lines which are HLA-B44 positive were examined.

All cell lines tested were labelled with ^{51}Cr for one

15 hour, at 37°C. They were then added in Iscove's medium plus 2% fetal calf serum, to various numbers of the two CTL clones discussed in example 14. The amount of ^{51}Cr released was measured after four hours. Controls were also used, as indicated in figure 13. Note that the cell line LB33-MEL was

20 incubated with IFN-γ (50 U/ml), for 48 hours before the assay.

The results of these experiments are shown in figure 13. CTL clones LB816-CTL-340 A/1 and LB822-CTL-346 A/1 lysed tumor cells expressing MAGE-3, but did not lyse LB33-EBV B cells which did not express the MAGE gene. The CTL clone LB822-346

25 A/1 lysed the HLA-B*4403$^+$ tumor cell line MZ2-MEL, which expresses MAGE-3, but did not lyse the antigen loss variant MZ2-MEL.61.2D$^-$.

Example 17

As a final test to determine if the peptide of SEQ ID NO:

30 17, in complexes with HLA-B44, stimulated CTLs, experiments were carried out to determine if tumor necrosis factor release was stimulated.

First, COS-7 cells were transfected by cDNA encoding MAGE-3 following Gaugler, et al, J. Exp. Med 179:921-930

35 (1994), in the expression vector pcDNA-1/AMP, and one of HLA-B*4402 cDNA cloned into vector pcDNA3, or HLA-B*4403 cDNA cloned into pcDNA1/AMP. The DEAE dextran chloroquine method

of Aruffo, Proc. Natl. Acad. Sci. USA 84:3365-3369 (1987) was used.

Transfectants were incubated for 24 hours, at 37°C, then 3000 CTLs/well were added. Materials were incubated for 18 hours, at 37°C. Supernatants were then collected, and TNF content was determined by testing the cytolytic effect on TNF sensitive WEHI-16 clone 13 cells, following Espevik et al, J. Immunol. Meth 95:99-105 (1986).

Table 2, which follows, shows the results, wherein TNF release is expressed in pg/ml. LB33-MEL and LB494-MEL were incubated with IFNγ at 100 U/ml for 24 hours prior to the assay. Tumor cell lines LB33-MEL, LB494-MEL, and MZ2-MEL were also tested. These cell lines all express MAGE-3 cDNA, and are either HLA-B*4402⁺ (LB33-MEL, LB494-MEL), or HLA-B*4403⁺ (MZ2-MEL). Hence, no transfection was necessary for these cells. The results show that TNF was released. Hence, one concludes that SEQ ID NO: 17 is being presented by HLA-B44 MHC molecules, and these complexes provoke CTL activity.

Table 2. TNF production of anti-MAGE-3.B44 CTL clones

	CTL Clones	Stimulator cells	TNF(pg/ml)
A	LB816-CTL-340A/1 (B4402)	COS	0.7
		COS+MAGE-3	0.6
		COS+HLA-B4402	0.5
		COS+HLA+B4402+MAGE-3	33.7
		LB33-MEL (B4402, MAGE-3+++)	74.9
		LB494-MEL (B4402, MAGE-3⁺⁺⁺)	32.3
B	LB822-CTL-346A/1 (B4403)	COS	1.2
		COS+MAGE-3	1
		COS+HLA-B4403	1.2
		COS+HLA-B4403+MAGE-3	26.6
		MZ2-MEL (B4403, MAGE-3⁺⁺⁺)	67.3

Example 18

The results presented supra, taken with data presented by, e.g. Brichard, et al., Eur. J. Immunol. [CITE] (1995); Buseyne, et al., J. Virol 67: 694-702 (1993); Coulie, et al., Proc. Natl. Acad. Sci. USA 91: 2105-2109 (1994); DiBrino, et

al., Biochemistry 34: 10130-10138 (1995); Fleischhauer, et al., Tissue Antigens 44: 311-317 (1994); Khanna, et al., J. Exp. Med. 176: 169-176 (1992); Kita, et al., Hepatology 18: 1039-1044 (1993), show that peptides which bind to HLA-B44

5   molecules often contain Glu at position 2, and either Tyr or Phe in the last position. Analysis of the MAGE-3 sequence reveals that four peptide sequences satisfy these constraints, i.e.:

Gln Glu Glu Gly Pro Ser Thr Phe (SEQ ID NO: 20);

10   Met Glu Val Asp Pro Ile Gly His Leu Tyr (SEQ ID NO: 17);
Trp Glu Glu Leu Ser Val Leu Glu Val Phe (SEQ ID NO: 21);
Glu Glu Leu Ser Val Leu Glu Val Phe (SEQ ID NO: 22).

These peptides were synthesized using standard, solid phase techniques and were then used in experiments to

15   determine if they bound to HLA-B44 MHC molecules.

The cells used in the experiments were C1R cells which had been transformed with Epstein Barr Virus (EBV). These cells differ from other, EBV transformed B cells in that they express the MAGE-3 gene. They are also MHC negative, and

20   hence can serve as subjects for transfection with genes encoding MHC molecules. Samples were transfected with either of a cDNA molecule encoding HLA-B*4402, or with genomic DNA encoding HLA-B*4403 in accordance with Fleischhauer, et al., Tissue Antigens 44: 311-317 (1994) or Fleischhauer, et al., N.

25   Eng. J. Med 323: 1828-1822 (1990) both of which are incorporated by reference. The transfectants were grown in RPMI-1640 medium which had been supplemented with 10% fetal calf serum (FCS).

In the competitive assay, the peptide

30   Glu Glu Lys Leu Ile Val Val Ala Phe (SEQ ID NO: 18) was used, as it has been reported to bind HLA-B44. See, e.g., Coulie, et al., Proc. Natl. Acad. Sci. USA 92: 7976-7980 (1995), incorporated by reference. Cytolytic T cell clone LB33-CTL-159/5 was also reported to recognize and to lyse

35   cells presenting complexes of the peptide and HLA-B44 on their surface. This clone was used in the assay, together with a constant amount of SEQ ID NO: 18 together with varying amounts

27

of each of SEQ ID NOS: 20, 17, 21, 22 and the following control peptides:

Ser Glu Ile Trp Arg Asp Ile Asp Phe (SEQ ID NO: 3) and

5

Phe Leu Arg Gly Arg Ala Tyr Gly Leu (SEQ ID NO: 19)

To carry out the assay, the transfected C1R (C1R-B*4402 as C1R-B*4403) transformed cells were ^{51}Cr labelled for one hour, at 37°C, in the presence of anti-human class-I MHC monoclonal antibody W6/32 (30% w/v of culture medium of the

10 hybridoma cells), and washed three times. Labelled cells (1000 cells in 80 ul), were incubated in V bottom microwells for 30 minutes at 20°C, in serum free, X-VIVO 10 medium. Test peptides were added, at varying concentrations, as explained infra, followed by the addition of the peptide

15 Glu Glu Lys Leu Ile Val Val Ala Phe (SEQ ID NO: 18) at 50 ng/ml in 40 ul of X-VIVO 10 medium for C1R-B*4402, and 160 ng/ml for C1R-B*4403. This peptide is known to bind to both HLA-B*4402 and HLA-B*4403 allotypes. (See, e.g., Coulie, et al., Proc. Natl. Acad. Sci. USA 92: 7976-7980 (1995)). The

20 cells which had been stimulated with SEQ ID NO: 18 were incubated for 30 minutes at 20°C, and then washed in Iscove's medium containing 2% fetal calf serum. Then, CTL clone LB33-159/5, described by Coulie et al as recognizing and lysing cells which present complexes of SEQ ID NO: 18 and HLA-B44 on

25 their surface, were added at 20,000 cells in 150 ul of Iscove's medium supplemented with 10% human serum.

The competitive peptides were added at varying concentrations, until lysis by CTL 159/5 was inhibited by 50%. Lysis was determined using the formula described supra. When

30 no competitor peptide was used, lysis of C1R-B*4402 transformants was 58%, and that of C1R-B*4403 was 72%.

In the table that follows, SEQ ID NOS: 3 and 19 were used as controls. The former, i.e.:

Ser Glu Ile Trp Arg Asp Ile Asp Phe (SEQ ID NO: 3)

35 is derived from tyrosinase and is known to bind to both HLA-B*4402 and HLA-B*4403 (Brichard, et al., Eur. J. Immunol. (1995), while the latter, i.e.

Phe Leu Arg Gly Arg Ala Tyr Gly Leu (SEQ ID NO: 19) binds to HLA-B8 (Burrows, et al., J. Exp. Med. 171: 345-349 (1990)). The results, presented below, set forth the concentration, in uM, of peptide needed to inhibit lysis of
5   C1R-B44 cells sensitized with SEQ ID NO: 8.

Peptide	C1R-B*4402	C1R-B*4403
SEQ ID NO: 20	60	15
SEQ ID NO: 17	2	1
SEQ ID NO: 21	80	7
SEQ ID NO: 22	20	5
SEQ ID NO: 3	1	<1
SEQ ID NO: 19	>100	>100

15   As will be seen, all of SEQ ID NOS: 17, 20, 21 and 22 did inhibit lysis of C1R-B44 cells, which indicates that they bind to HLA-B44 molecules. The peptide of SEQ ID NO: 17 was the best binder for both HLA-B*B4402 and HLA-B*4403. This is shown in figure 14, wherein SEQ ID NOS: 17, 19 and 3 and are plotted
20   as a function of lysis versus concentration of competitor peptides.

In experiments not reported here, peptides homologous to SEQ ID NO: 17, and derived from the amino acid sequence of MAGE-1, 2, 4, 6 and 12 were also tested, and were found to
25   bind the HLA-B44 molecule.

Example 19

Once it was clear that various peptides did, in fact bind to HLA-B44 allotypes, it was of interest to determine if the peptides could be used to provoke cytolytic T cell clones
30   specific to the complexes of the peptide and the MHC molecule.

In order to determine this, adherent peripheral blood mononuclear cells were isolated from a donor, referred to as LB816, who did not suffer from cancer, and who was HLA-B*4402 positive.

35   The adherent cells were isolated by first securing a sample of peripheral blood mononuclear cells ("PBMCs" hereafter), using density gradient centrifugation. T-

lymphocytes were purified from the sample via rosetting, using 2-aminoethyl-isothiouronium bromide hydrobromide-treated sheep erythrocytes, in accordance with Mikamo, J. Immunol. Meth. 107: 189-196 (1988), incorporated by reference, and via labelling with anti-CD8 monoclonal antibodies coupled to magnetic microbeads, followed by sorting of the CD8$^+$ cells via passage in a magnetic field. The thus separated cells were frozen for storage. Any non-rosetting PBMCs were left to adhere for two hours at 37°C on NUNC tissue culture wells, with non-adherent cells being discarded.

The adherent cells were then cultured, for seven days, in the presence of IL-4 (50 U/ml), and GM-CSF (100 ng/ml), in RPMI medium supplemented with 10% FCS. GM-CSF and IL-4 are used in order to increase the proportion of dendritic cells in the culture. See Romani, et al., J. Exp. Med. 180: 83-93 (1994); Sallusto, et al., J. Exp. Med. 179: 1109-1118 (1994). When the cell populations were analyzed, those taken from donor LB816 were found, for the most part, to be CD11c$^+$ and CD14$^+$, while cells from donor LB822 were mostly CD11c$^+$ and CD14$^-$.

A total of from 5x10^5 to 5x10^6 of these antigen presenting cells were then incubated in 2 ml wells, for four hours at 37°C, in 400 ul of Iscove's medium supplemented with human ß2 microglobulin (2.5 ug/ml), and 50 ug/ml of SEQ ID NO: 17. Following this, the cells were irradiated at 50 Gy, and washed. These cells were then used as stimulator cells on the autologous CD8$^+$ cells discussed supra.

Six cultures of autologous CD8$^+$ wells were established. For each culture, 2x10^6 CD8$^+$ cells were combined with culture medium (Iscove's medium plus 10% human serum, L-arginine, L-asparagine, L-glutamine, 0.05 mM 2-mercaptoethanol, 1000 U/ml IL-6 and 5 ng/ml IL-12). On days 7 and 14, these responder cells wee stimulated with the stimulator cells. To do this, on day 7 the PBMCs (5x10^6) were left to adhere for 2 hours, at 37°C in 400 ul of Iscove's medium containing ß2 microglobulin, and the peptide of SEQ ID NO: 17, as described supra. The adherent cells were irradiated and washed, also as described

<u>supra</u>. The responder lymphocytes (i.e., the CD8⁺ cells), in culture medium which had been augmented with 10 U/ml of IL-2 and 5 ng/ml of IL-7 were added. On day 14, the lymphocytes were restimulated with PBMCs which had been pulsed with peptide. In this restimulation, 2×10^7 PBMCs per ml were incubated for two hours at 20°C in Iscove's medium with ß2 microglobulin, and the peptide of SEQ ID NO: 17, as described supra. Again, these PBMCs were irradiated, washed, and resuspended at 2×10^6 cells/ml in culture medium supplemented with IL-2 and IL-7, and, again as described, were added to the responders.

At day 20 the lytic activity was assessed on LB816 cells (HLA-B*4402 positive cells), which had been sensitized with SEQ ID NO: 17. The lytic activity was measured using the ^{51}Cr release assay as described supra. The results are shown in figure 15A. Effector cells were incubated with unlabelled K562 target cells (50,000 cells/well) for 45 minutes, in order to inhibit lysis by NK-like effectors. ^{51}Cr-labelled target cells (1000 cells/well) were incubated with 1 µM of SEQ ID NO: 17 (filled in circle) or without it (open circle). ^{51}Cr release was measured after 4 hours. Only one of the six autologous CD8⁺ culture preferentially lysed the target cells.

Similarly, HLA-B*4403 positive cells (LB822), were tested. Results, presented in figure 15B, show that one out of five cultures tested preferentially lysed the cells.

Based upon these results, it was concluded that the frequency of precursors of anti-SEQ ID NO: 17/HLA-B44 cells was on the order of 1 out of every 10^7 CD8⁺ lymphocytes.

The lymphocytes of the positive cultures were cloned by limiting dilution. Specifically, on day 21, these cells were cultured in culture medium supplemented with 50 U/ml of IL-2, and 5 U/ml of IL-4. The LB816 cells were stimulated with irradiated C1R-B*4402 cells (150 Gy, 8000 cells/wells), which had been incubated for one hour at 20°C with 1 ug/ml of SEQ ID NO: 17, and washed. The LB822 clones were stimulated with irradiated MZ2-MEL cells which are HLA-B*4403 positive melanoma cell (100 Gy, 8000 cells per well). Irradiated

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allogeneic LG2-EBV cells (100 Gy, 20,000 cells/well) were added as feeder cells. Microcultures were stimulated each week. CTL clones LB816-CTL-340/1 and LB822-CTL-346/8 were isolated in this fashion.

5 **Example 20**

The CTL clones discussed supra were stimulated each week in 2 ml wells with 2×10^5 irradiated ClR-B*4402 or ClR-B*4403 cells, which had been incubated for one hour at 20°C with 1 ug/ml of the peptide of SEQ ID NO: 17, then washed, and with

10 10^6 irradiated LG2-EBV cells, all in culture medium supplemented with IL-2 and IL-4, as before.

In order to confirm that the CTL clones thus isolated recognized complexes of SEQ ID NO: 17, and both allotypes of HLA-B44, 10,000 cells of each CTL clone (per well) were

15 combined with 1000 cells (per well), of ^{51}Cr labelled lymphoblastoid B cells (LB33-EBV, which is HLA-B*4402 positive), which had been incubated, for 30 minutes, at 20°C in serum free medium, with a varying concentration of SEQ ID NO: 17, and then washed. Release of ^{51}Cr was measured after

20 four hours, using the methods described herein.

The results, shown in figure 16, demonstrate that, for CTL 340/1, half maximal lysis was obtained at approximately 40 nM, while half maximal lysis was obtained for CTL 346/8 at about 100 nM.

25 In experiments only summarized here, a peptide homologous to SEQ ID NO: 17 but for a difference at position 9 (where leucine, was replaced by valine was tested. This peptide is from MAGE-6. It bound efficiently to both HLA-B44 subtypes, although the specific CTL clones being considered did not

30 recognize it. Thus, for purposes of binding HLA-B44 molecules, the ninth position was not essential.

Example 21

A further set of experiments were carried out to determine if SEQ ID NO: 17 was naturally presented on cells

35 which express the MAGE-3 gene.

To determine this, COS-7 cells (15,000 per well) were cotransfected with 50 ng of pcDNAI/Amp containing cDNA for

MAGE-3, and either 50 ng of pcDNAI/Amp containing cDNA for HLA-B*4403, or 50 ng of pcDNA3/Amp containing cDNA for HLA-B*4402. The cotransfection was carried out in accordance with the well known protocol of Seed, et al., Proc. Natl. Acad. Sci. USA 84: 3365-3369 (1987). Also, NA8-MEL melanoma cells were cotransfected. These cells do not express either of HLA-B44 or MAGE-3 molecules. To cotransfect NA8-MEL (30,000 cells per well) 100 ng samples of the plasmids given supra were used, using LIPOFECTAMINE.

Following cotransfection, cells were incubated for 36 hours at 37°C, and then tested for their ability to stimulate production of tumor necrosis factor (TNF), in the presence of the CTL clones 340/1 and 346/8, discussed supra. TNF production was determined following Lehmann et al., Eur. J. Immunol. 25: 340-347 (1995), incorporated by reference, but discussed briefly here. In these experiments, 3000 CTLs were added in 100 ul of Iscove's medium supplemented with 10% human serum, and 25 U/ml of IL-2. After 20 hours, supernatant was collected and TNF content determined by testing its cytotoxicity on WEHI 164c13 cells, in accordance with Espevik et al., J. Immunol. Meth. 95: 99-105 (1986). In these experiments, summarized in figure 17, controls were prepared using single transfections (either MAGE-3 or HLA-B*4402 or HLA-B*4403).

Figure 17 shows that cells cotransfected with the two constructs stimulated TNF release, while cells transfected with one plasmid did not. This result was observed with both cell types, and both CTLs. The recognition of MAGE-3/HLA-B44 complexes did not require the high copy number provided by COS-7 cells, as the NA8-MEL work shows.

Example 22

The CTLs described supra, i.e., 340/1 and 346/8, were then tested for their ability to lyse HLA-B44 presenting tumor cells. The cells used included lines known to express MAGE-3, including LB33-MEL-A-1, LB373-MEL, LB494-MEL, LB831-BLC, LG2-MEL and MZ2-MEL.43, which are melanoma cells except for LB831-BLC, which is a bladder cancer derived cell line. Cell line

MZ2-MEL.61.2 is a melanoma cell line which has lost expression
of MAGE-3. In figure 18, which is discussed _infra_, this cell
is depicted by open ("O") circles, rather than closed ("O")
circles. All cells, with the exception of MZ2-MEL.43, MZ2-
MEL.61.2, and LG2-MEL were incubated, over 48 hours, in the
presence of 50 U/ml of IFN-γ, prior to use in a ^{51}Cr release
assay. In each case, the expression of HLA-B*4402, or HLA-
B*4403, and MAGE-3 was tested, using RT-PCR methodologies not
repeated here.

The results, set forth in figure 18, show that the CTLs
did recognize and lyse these naturally MAGE-3/HLA-B44
expressing cells.

The foregoing experiments describe isolated nucleic acid
molecules coding for a tumor rejection antigen precursor, a
"TRAP" molecule. The protein molecule for which these code is
processed intracellularly in a manner which leads to
production of at least one tumor rejection antigen, or "TRA",
which is presented by HLA-B44 molecules. While it has been
observed previously that HLA-B44 molecules present peptides
derived from tyrosinase, the nucleic acid molecules of the
invention do not code for tyrosinase, and the TRAs are not
tyrosinase derived.

The tumor rejection antigens of the invention are
isolated nonapeptides which have a Glu residue at the 2nd
position, and a Phe or Tyr residue at the 9th or 10th
position. Especially preferred are peptides which satisfy the
following formula:

Xaa Glu Xaa Asp Xaa Xaa Xaa Xaa Xaa Tyr (SEQ ID NO: 23)
or

Xaa Glu Xaa Val Xaa Xaa Xaa Xaa Xaa Tyr (SEQ ID NO: 24)
wherein Xaa is any amino acid. These generic formulas embrace
SEQ ID NO: 17, as well as homologous peptides derived from
different MAGE proteins which, as has been discussed, supra,
also bind to HLA-B44 molecules. These homologous include:

Lys Glu Ala Asp Pro Thr Gly His Ser Tyr (SEQ ID NO: 25);
Val Glu Val Val Pro Ile Ser His Leu Tyr (SEQ ID NO: 26);
Lys Glu Val Asp Pro Ala Ser Asn Thr Tyr (SEQ ID NO: 27);

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Met Glu Ala Asp Pro Thr Ser Asn Thr Tyr (SEQ ID NO: 28)

Met Glu Val Asp Pro Ile Gly His Val Tyr (SEQ ID NO: 29);
and

Val Glu Val Val Arg Ile Gly His Leu Tyr (SEQ ID NO: 30).
These correspond to peptide sequences which are homologous to
SEQ ID NO: 17, in that they are found at the corresponding
positions of MAGE-1, 2, 4, 5, 6 and 12, respectively. Their
ability to bind to HLA-B44 molecules renders them useful,
inter alia, in identifying cells which present HLA-B44
molecules in their surfaces.

The peptides of the invention are similar to the peptide
disclosed in Serial No. 08/233,305, so-assigned to the
assignee of the subject application, i.e.:

Ser Glu Ile Trp Arg Asp Ile Asp Phe

(SEQ ID NO: 3)

Khanna, et al., supra, teaches a decamer, i.e.:

Glu Glu Asn Leu Leu Asp Phe Val Arg Phe

(SEQ ID NO: 4)

but does not discuss how modification of the decamer could
lead to an effective nonamer.

The invention thus involves tumor rejection antigens
which bind to HLA-B44 molecules, and then provoke lysis by
CTLs.

As indicated, the complexes of TRA and HLA molecule
provoke a cytolytic T cell response, and as such isolated
complexes of the tumor rejection antigen and an HLA-B44
molecule are also encompassed by the invention, as are
isolated tumor rejection antigen precursors coded for by the
previously described nucleic acid molecules. Given the
binding specificity, the peptides may also be used, simply to
identify HLA-B44 positive cells.

The invention as described herein has a number of uses,
some of which are described herein. First, the identification
of a tumor rejection antigen which is specifically presented
by an HLA-B44 molecule, as well as a nucleic acid molecule

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coding for its parallel tumor rejection antigen precursor permits the artisan to diagnose a disorder characterized by expression of the TRAP. These methods involve determining expression of the TRAP gene, and/or TRAs derived therefrom, such as TRA presented by HLA molecules. Other TRAs may also be derived from the TRAPs of the invention and presented by different HLA molecules. In the former situation, such determinations can be carried out via any standard nucleic acid determination assay, including the polymerase chain reaction, or assaying with labelled hybridization probes. In the latter situation, assaying with binding partners for complexes of TRA and HLA, such as antibodies, is especially preferred.

The isolation of the TRAP gene also makes it possible to isolate the TRAP molecule itself, especially TRAP molecules containing the amino acid sequence of SEQ ID NO: 1. Fragments of peptides of these isolated molecules when presented as the TRA, or as complexes of TRA and HLA-B44, may be combined with materials such as adjuvants to produce vaccines useful in treating disorders characterized by expression of the TRAP molecule. In addition, vaccines can be prepared from cells which present the TRA/HLA complexes on their surface, such as non-proliferative cancer cells, non-proliferative transfectants, etcetera. In all cases where cells are used as a vaccine, these can be cells transfected with coding sequences for one or both of the components necessary to prove a CTL response, or be cells which express both molecules without transfection. Further, the TRAP molecule, its associated TRAs, as well as complexes of TRA and HLA, may be used to produce antibodies, using standard techniques well known to the art.

When "disorder" is used herein, it refers to any pathological condition where the tumor rejection antigen precursor is expressed. An example of such a disorder is cancer, melanoma in particular.

Therapeutic approaches based upon the disclosure are premised on a response by a subject's immune system, leading

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to lysis of TRA presenting cells, such as cells presenting the relevant HLA molecule. One such approach is the administration of CTLs specific to the complex to a subject with abnormal cells of the phenotype at issue. it is within the skill of the artisan to develop such CTLs in vitro. Specifically, a sample of cells, such as blood cells, are contacted to a cell presenting the complex and capable of provoking a specific CTL to proliferate. The target cell can be a transfectant, such as a COS cell of the type described supra. These transfectants present the desired complex on their surface and, when combined with a CTL of interest, stimulate its proliferation. COS cells, such as those used herein are widely available, as are other suitable host cells.

To detail the therapeutic methodology, referred to as adoptive transfer (Greenberg, J. Immunol. 136(5): 1917 (1986); Reddel et al., Science 257: 238 (7-10-92); Lynch et al., Eur. J. Immunol. 21: 1403-1410 (1991); Kast et al., Cell 59: 603-614 (11-17-89)), cells presenting the desired complex are combined with CTLs leading to proliferation of the CTLs specific thereto. The proliferated CTLs are then administered to a subject with a cellular abnormality which is characterized by certain of the abnormal cells presenting the particular complex. The CTLs then lyse the abnormal cells, thereby achieving the desired therapeutic goal.

The foregoing therapy assumes that at least some of the subject's abnormal cells present the HLA/TRA complex. This can be determined very easily, as the art is very familiar with methods for identifying cells which present a particular HLA molecule, as well as how to identify cells expressing DNA containing the indicated sequences. Once isolated, such cells can be used with a sample of a subject's abnormal cells to determine lysis in vitro. If lysis is observed, then the use of specific CTLs in such a therapy may alleviate the condition associated with the abnormal cells. A less involved methodology examines the abnormal cells for HLA phenotyping, using standard assays, and determines expression via amplification using, e.g., PCR.

Adoptive transfer is not the only form of therapy that is available in accordance with the invention. CTLs can also be provoked in vivo, using a number of approaches. One approach, i.e., the use of non-proliferative cells expressing the

5   complex, has been elaborated upon supra. The cells used in this approach may be those that normally express the complex, such as irradiated melanoma cells or cells transfected with one or both of the genes necessary for presentation of the complex. Chen et al., Proc. Natl. Acad. Sci. USA 88: 110-114

10 (January, 1991) exemplifies this approach, showing the use of transfected cells expressing HPVE7 peptides in a therapeutic regime. Various cell types may be used. Similarly, vectors carrying one or both of the genes of interest may be used. Viral or bacterial vectors are especially preferred. In these

15 systems, the gene of interest is carried by, e.g., a Vaccinia virus or the bacteria BCG, and the materials de facto "infect" host cells. The cells which result present the complex of interest, and are recognized by autologous CTLs, which then proliferate. A similar effect can be achieved by combining

20 the tumor rejection antigen or the precursor itself with an adjuvant to facilitate incorporation into cells which present the HLA molecule of interest. The TRAP is processed to yield the peptide partner of the HLA molecule while the TRA is presented without the need for further processing.

25   Other aspects of the invention will be clear to the skilled artisan and need not be repeated here.

The terms and expressions which have been employed are used as terms of description and not of limitation, and there is no intention in the use of such terms and expressions of

30 excluding any equivalents of the features shown and described or portions thereof, it being recognized that various modifications are possible within the scope of the invention.

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    (ii) TITLE OF INVENTION: Tumor Rejection Antigens
                            Presented By HLA-B44 Molecules,
                            And Uses Thereof

    (iii) NUMBER OF SEQUENCES: 30

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        (C) OPERATING SYSTEM: PC-DOS

        (D) SOFTWARE: Wordperfect

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10

(2)　INFORMATION FOR SEQ ID NO: 1:

　　(i)　SEQUENCE CHARACTERISTICS:

　　　　(A)　LENGTH:　1896 base pairs

　　　　(B)　TYPE:　nucleic acid

5　　　　(C)　STRANDEDNESS: single

　　　　(D)　TOPOLOGY:　linear

　　(xi)　SEQUENCE DESCRIPTION:　SEQ ID NO: 1:

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10  GCGGCGGTGG CGGAGGCGGA CACATTGGCG TGAGACCTGG GAGTACGTTG TGCCAAATCA   60
    TTGCCACTTG CCACATGAGT GTAAATGATG GCGGATGCAA GTATGTCCTC TGCCGATGGG  120
    AAAAGCGATT ATGGCCTGCG AAGGTGACAG CCATTATTCT GTAACTTCAG GACTTAGAAA  180
    TGACTTTCGG GTGACAAGTA AAATCTTGAT CAGGAGATAC CTAGGATTTG CTTCAGTGAA  240
    ATAATTGAGC CAGAACACGG TTGGCACTGA TTCTCGTTCC CCATTTAATG GGGTTTTGGT  300
15  CTAGTGCTTC CAAGGTTACA CTTCCAGAAA TGTCTTTTTT TTTTCACACT AAAAAAAAAA  360
    AAAAGAATCA GCTGTAAAAA GGCATGTAAG GCTGTAACTC AAGGAAAGAT CTGGCAAGCA  420
    GCCCTGTGAT AGTAAATTAT GGTCGTGTTC AGGGAATGCT TTCCAGCAAT TCAGTAGACA  480
    GTGCTCAGCT GCAATGCAAA AGCCCAGGTC CTTGTCTTTG TCTGCCACTG GCCTCTCATG  540
    CCTCAGTTTC CCCATCTGTG AAACAATGGG GATTGGACCA AATATCTGAA ATCCCATGGT  600
20  TATAGGCCTT CAGGATTACC TGCTGCATTT GTGCTAAAGT TTGCCACTGT TTCTCACTGT  660
    CAGCTGTTGT AATAACAAGG ATTTTCTTTT GTTTTAAATG TAGGTTTTGG CCCGAACCGC  720
    GACTTCAACA AAAAATAAGA GAAGAAAGGA ATATTTTCTA GCTGTGCAAA TCCTCTCCCT  780
    AGAGGAAAAG TTAATTGTTG TGTTGTTTTA ATACTGTTTT TTCCCGTGTA GATTTCTGAT  840
    ACTTCAATCC CCTACTCCCC CAAAACAGTT GAAGCCCAGC CCACTCTTAA TGGGCTTATT  900
25  CACCATTTGT GTAATTCATT AATGCTCATA ATAACCTCAT GAGAAAGCAA CTAGTTTGAT  960
    TTTATGTCAG TTTGGAAGCT GAAGATCCAA ACGAGGCATT CTGTGAGATC TATGGAGAGA 1020
    TTGGTACAAA CACTGAATAC ATGTAAATTA TACTCAGGGT AGACCCTATT TGTGGTTAAA 1080
    ATAGGGATAT TTCCTTTTTT TTTTTTTTTT TTTTGACTGT TTCTTAATCA GTGCCATGCC 1140
    AGGAAAATAG GGATGTTTCC TTCCCAGAGA TCTGTGTGTC TTTTTTCAGA AACGTCTGTG 1200
30  ACAGGCCCAT CAATTTTGAA ATATTTGGTT TTTGAGCCTG TCACTCTAAA CCAGCGTTTA 1260
    ACGTTCAAAA GGCAAATAAC TGATGACCAG GCGGCACATT GTTCTGCTCC GTGAGTGTCT 1320
    GGCACTGGGA AAGGTGTAGA TTGTCTAGAA TGACAGCAAT TCCGACGCCC CAGTCAGTCC 1380
    TGCGTGATTG TGGCGAGGGC GCGTCTGGCA CCGGGAAGGT GTAGATCATC TAGAATGACG 1440
    GCGATTCCGA CGCCCCGGTC AGTCCTGCGT GATTGGCGAG GGTGCATCTG TCGTGAGAAT 1500
35  TCCCAGTTCT GAAGAGAGCA AGGAGACTGA TCCCGCGTAG TCCAAGGCAT TGGCTCCCCT 1560
    GTTGCTCTTC CTTGTGGAGC TCCCCCTGCC CCACTCCCTC CTGCCTGCAT CTTCAGAGCT 1620
    GCCTCTGAAG CTCGCTTGGT CCCTAGCTCA CACTTTCCCT GCGGCTGGGA AGGTAATTGA 1680
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ATACTCGAGT TTAAAAGGAA AGCACATCCT TTTAAACCAA AACACACCTG CTGGGCTGTA 1740
AACAGCTTTT AGTGACATTA CCATCTACTC TGAAAATCTA ACAAAGGAGT GATTTGTGCA 1800
GTTGAAAGTA GGATTTGCTT CATAAAAGTC ACAATTTGAA TTCATTTTTG CTTTTAAATC 1860
CAGCCAACCT TTTCTGTCTT AAAAGGAAAA AAAAAA                            1896
```

(2)   INFORMATION FOR SEQ ID NO: 2:
    (i)   SEQUENCE CHARACTERISTICS:
        (A)   LENGTH: amino acid residues
        (B)   TYPE: 9 amino acids
        (D)   TOPOLOGY: linear
    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Glu Glu Lys Leu Ile Val Val Leu Phe
            5

(2)   INFORMATION FOR SEQ ID NO: 3:
    (i)   SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 9 amino acids
        (B) TYPE: amino acid
        (D) TOPOLOGY: linear
    (ii) MOLECULE TYPE: protein
    (ix) FEATURE:
        (A) NAME/KEY: HLA-B44 binding peptide
    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

Ser Glu Ile Trp Arg Asp Ile Asp Phe
            5

(2)   INFORMATION FOR SEQ ID NO: 4:
    (i)   SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 10 amino acids
        (B) TYPE: amino acid
        (D) TOPOLOGY: linear

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```
        (ii) MOLECULE TYPE: protein
        (ix) FEATURE:
             (A) NAME/KEY:  Khanna peptide
        (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:
5


    Glu Glu Asn Leu Leu Asp Phe Val Arg Phe
                      5                   10



10   (2)   INFORMATION FOR SEQ ID NO: 5:
           (i)   SEQUENCE CHARACTERISTICS:
                 (A) LENGTH: 18 base pairs
                 (B) TYPE:  nucleic acid
                 (D) TOPOLOGY:  linear
15   (ii) MOLECULE TYPE: nucleic acid
        (ix) FEATURE:
             (A) NAME/KEY:  PCR primer
        (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:


20   GCCGGAGTAT TGGGACGA                                18



     (2)   INFORMATION FOR SEQ ID NO: 6:
           (i)   SEQUENCE CHARACTERISTICS:
25               (A) LENGTH:      17 base pairs
                 (B) TYPE:        nucleic acid
                 (D) TOPOLOGY:  linear
        (ii) MOLECULE TYPE: nucleic acid
        (ix) FEATURE:
30           (A) NAME/KEY:  PCR primer
        (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:


     GGCCGCCTCC CACTTGC                                17


35
```

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(2)   INFORMATION FOR SEQ ID NO: 7:

     (i)SEQUENCE CHARACTERISTICS:

        (A) LENGTH: 19 base pairs

        (B) TYPE: nucleic acid

5   (D) TOPOLOGY: linear

    (ii)MOLECULE TYPE: nucleic acid

    (ix)FEATURE:

        (A) NAME/KEY: PCR primer

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

10

GGAGTATTGG GACCGGAAG   19

(2)   INFORMATION FOR SEQ ID NO: 8:

15   (i)   SEQUENCE CHARACTERISTICS:

        (A) LENGTH: 18 base pairs

        (B) TYPE: nucleic acid

        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: nucleic acid

20   (ix) FEATURE:

        (A) NAME/KEY: PCR primer

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

GGCCGCCTCC CACTTGT   18

25

(2)   INFORMATION FOR SEQ ID NO: 9:

    (i)   SEQUENCE CHARACTERISTICS:

        (A) LENGTH: 18 base pairs

30   (B) TYPE: nucleic acid

        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: nucleic acid

    (ix) FEATURE:

        (A) NAME/KEY: PCR primer

35   (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

CGCCACGAGT CCGAGGAT   18

44

(2)   INFORMATION FOR SEQ ID NO: 10:
         (i)   SEQUENCE CHARACTERISTICS:
                  (A) LENGTH: 18 base pairs
                  (B) TYPE: nucleic acid
                  (D) TOPOLOGY: linear
         (ii) MOLECULE TYPE: nucleic acid
         (ix) FEATURE:
                  (A) NAME/KEY: PCR primer
         (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

CCTTGCCGTC GTAGGCTA   18

(2)   INFORMATION FOR SEQ ID NO: 11:
         (i)   SEQUENCE CHARACTERISTICS:
                  (A) LENGTH: 18 base pairs
                  (B) TYPE: nucleic acid
                  (D) TOPOLOGY: linear
         (ii) MOLECULE TYPE: nucleic acid
         (ix) FEATURE:
                  (A) NAME/KEY: PCR primer
         (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

CGCCACGAGT CCGAGGAA   18

(2)   INFORMATION FOR SEQ ID NO: 12:
         (i)   SEQUENCE CHARACTERISTICS:
                  (A) LENGTH: 18 base pairs
                  (B) TYPE: nucleic acid
                  (D) TOPOLOGY: linear
         (ii) MOLECULE TYPE: nucleic acid
         (ix) FEATURE:
                  (A) NAME/KEY: PCR primer
         (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

CCTTGCCGTC GTAGGCGT   18

(2)   INFORMATION FOR SEQ ID NO: 13:
        (i)   SEQUENCE CHARACTERISTICS:
                (A) LENGTH: 19 base pairs
                (B) TYPE: nucleic acid
                (D) TOPOLOGY: linear
        (ii) MOLECULE TYPE: nucleic acid
        (ix) FEATURE:
                (A) NAME/KEY: PCR primer
        (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

CCGAGTGAAC CTGCGGAAA   19

(2)   INFORMATION FOR SEQ ID NO: 14:
        (i)   SEQUENCE CHARACTERISTICS:
                (A) LENGTH: 19 base pairs
                (B) TYPE: nucleic acid
                (D) TOPOLOGY: linear
        (ii) MOLECULE TYPE: nucleic acid
        (ix) FEATURE:
                (A) NAME/KEY: PCR primer
        (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

GGTCGCAGCC ATACATCCA   19

(2)   INFORMATION FOR SEQ ID NO: 15:
        (i)   SEQUENCE CHARACTERISTICS:
                (A) LENGTH: 19 base pairs
                (B) TYPE: nucleic acid
                (D) TOPOLOGY: linear
        (ii) MOLECULE TYPE: nucleic acid
        (ix) FEATURE:
                (A) NAME/KEY: PCR primer
        (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

TACAAGCGCC AGGCACAGG   19

46

(2)   INFORMATION FOR SEQ ID NO: 16:
         (i)   SEQUENCE CHARACTERISTICS:
                (A) LENGTH: 18 base pairs
                (B) TYPE: nucleic acid
                (D) TOPOLOGY: linear
         (ii) MOLECULE TYPE: nucleic acid
         (ix) FEATURE:
                (A) NAME/KEY: PCR primer
         (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

CTCCAGGTAG GCTCTGTC   18

(2)   INFORMATION FOR SEQ ID NO: 17:
         (i) SEQUENCE CHARACTERISTICS:
                (A) LENGTH: 10 amino acids
                (B) TYPE: amino acid
                (D) TOPOLOGY: linear
         (ii) MOLECULE TYPE: protein
         (ix) FEATURE:
         (A)   NAME/KEY: Mage-3 peptide
         (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

Met Glu Val Asp Pro Ile Gly His Leu Tyr
                        5   10

(2)   INFORMATION FOR SEQ ID NO: 18:
         (i)   SEQUENCE CHARACTERISTICS:
                (A) LENGTH: 9 amino acids
                (B) TYPE: amino acid
                (D) TOPOLOGY: linear
         (ii) MOLECULE TYPE: protein
         (ix) FEATURE:
                (A)   NAME/KEY:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

Glu Glu Lys Leu Ile Val Val Ala Phe
                    5

(2)   INFORMATION FOR SEQ ID NO: 19:
      (i)   SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 9 amino acids
            (B) TYPE: amino acid
            (D) TOPOLOGY: linear
      (ii) MOLECULE TYPE: protein
      (ix) FEATURE:
            (A) NAME/KEY:
      (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19:

Phe Leu Arg Gly Arg Ala Tyr Gly Leu
                    5

(2)   INFORMATION FOR SEQ ID NO: 20:
      (i)   SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 8 amino acids
            (B) TYPE: amino acid
            (D) TOPOLOGY: linear
      (ii) MOLECULE TYPE: protein
      (ix) FEATURE:
            (A) NAME/KEY: Mage-3 peptide
      (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 20

Gln Glu Glu Gly Pro Ser Thr Phe
                    5

(2)   INFORMATION FOR SEQ ID NO: 21:
      (i)   SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 10 amino acids

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```
                    (B)  TYPE: amino acid
                    (D)  TOPOLOGY: linear
            (ii)  MOLECULE TYPE: protein
            (ix)  FEATURE:
  5              (A)  NAME/KEY: Mage-3 peptide
            (xi)  SEQUENCE DESCRIPTION: SEQ ID NO: 21:


    Trp Glu Glu Leu Ser Val Leu Glu Val Phe
                        5                   10
 10


    (2)    INFORMATION FOR SEQ ID NO: 22:
            (i)   SEQUENCE CHARACTERISTICS:
                    (A)  LENGTH: 9 amino acids
 15                 (B)  TYPE: amino acid
                    (D)  TOPOLOGY: linear
            (ii)  MOLECULE TYPE: protein
            (ix)  FEATURE:
                    (A)  NAME/KEY: Mage-3 peptide
 20     (xi)  SEQUENCE DESCRIPTION: SEQ ID NO: 22:


    Glu Glu Leu Ser Val Leu Glu Val Phe
                        5


 25
    (2)    INFORMATION FOR SEQ ID NO: 23:
            (i)   SEQUENCE CHARACTERISTICS:
                    (A)  LENGTH: 10 amino acids
                    (B)  TYPE: amino acid
 30                 (D)  TOPOLOGY: linear
            (ii)  MOLECULE TYPE: protein
            (ix)  FEATURE:
                    (A)  NAME/KEY: HLA-B44 motif
            (xi)  SEQUENCE DESCRIPTION: SEQ ID NO: 23:
 35
    Xaa Glu Xaa Asp Xaa Xaa Xaa Xaa Xaa Tyr
                        5                   10
```

(2)   INFORMATION FOR SEQ ID NO: 24:

    (i)   SEQUENCE CHARACTERISTICS:

      (A) LENGTH: 10 amino acids

      (B) TYPE: amino acid

      (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: protein

    (ix) FEATURE:

      (A) NAME/KEY: HLA-B44 motif

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 24:

Xaa Glu Xaa Val Xaa Xaa Xaa Xaa Xaa Tyr

                    5   10

(2)   INFORMATION FOR SEQ ID NO: 25:

    (i) SEQUENCE CHARACTERISTICS:

      (A) LENGTH: 10 amino acids

      (B) TYPE: amino acid

      (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: protein

    (ix) FEATURE:

      (A) NAME/KEY: MAGE-1/HLA-B44

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 25:

Lys Glu Ala Asp Pro Thr Gly His Ser Tyr

                    5   10

(2)   INFORMATION FOR SEQ ID NO: 26:

    (i)   SEQUENCE CHARACTERISTICS:

      (A) LENGTH: 10 amino acids

      (B) TYPE: amino acid

      (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: protein

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(ix) FEATURE:

    (A) NAME/KEY: Mage-2/HLA-B44

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 26:

Val Glu Val Val Pro Ile Ser His Leu Tyr
                    5   10

(2) INFORMATION FOR SEQ ID NO: 27:

    (i) SEQUENCE CHARACTERISTICS:

        (A) LENGTH: 10 amino acids

        (B) TYPE: amino acid

        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: protein

    (ix) FEATURE:

        (A) NAME/KEY: Mage-4/HLA-B44

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 27:

Lys Glu Val Asp Pro Ala Ser Asn Thr Tyr
                    5   10

(2) INFORMATION FOR SEQ ID NO: 28:

    (i) SEQUENCE CHARACTERISTICS:

        (A) LENGTH: 10 amino acids

        (B) TYPE: amino acid

        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: protein

    (ix) FEATURE:

        (A) NAME/KEY: Mage-5/HLA-B44

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 28:

Met Glu Ala Asp Pro Thr Ser Asn Thr Tyr
                    5   10

```
(2)   INFORMATION FOR SEQ ID NO: 29:
      (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 10 amino acids
            (B) TYPE: amino acid
            (D) TOPOLOGY: linear
      (ii) MOLECULE TYPE: protein
      (ix) FEATURE:
            (A) NAME/KEY: Mage-6/HLA-B44
      (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 29:
```

```
Met Glu Val Asp Pro Ile Gly His Val Tyr
                  5                   10
```

```
(2)   INFORMATION FOR SEQ ID NO: 30:
      (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 10 amino acids
            (B) TYPE: amino acid
            (D) TOPOLOGY: linear
      (ii) MOLECULE TYPE: protein
      (ix) FEATURE:
            (A) NAME/KEY: Mage-12/HLA-B44
      (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 30:
```

```
Val Glu Val Val Arg Ile Gly His Leu Tyr
                  5                   10
```

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We claim:

1. Isolated peptide which binds to HLA-B44 molecules and consists of amino acid sequence:

Xaa Glu Xaa Asp Xaa Xaa Xaa Xaa Xaa Tyr (SEQ ID NO: 23)

or amino acid sequence

Xaa Glu Xaa Val Xaa Xaa Xaa Xaa Xaa Tyr (SEQ ID NO: 24),

with the proviso that said peptide is not SEQ ID NO: 17.

2. The isolated peptide of claim 1, selected from the group consisting of: SEQ ID NO: 25, SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 29, and SEQ ID NO: 30.

3. Method for identifying an HLA-B44 positive cell in a sample, comprising contacting said sample with the isolated peptide of claim 1, and determining binding of said peptide as a determination of an HLA-B44 positive cell in said sample.

4. Isolated, cytolytic T cell clone which is specific for complexes of SEQ ID NO: 17 and an HLA-B44 molecule.

5. The isolated, cytolytic T cell clone of claim 4, wherein said HLA-B44 molecule is an HLA-B*4402 molecule.

6. The isolated, cytolytic T cell clone of claim 4, wherein said HLA-B44 molecule is an HLA-B*4403 molecule.

FIG. 1C

FIG. 1B

FIG. 1A

FIG. 2

FIG. 3

Stimulator Cells	TNF Released by CTL 159/5 (pg/ml)
	20 40 60 80 100
LB33-MEL.c1	████████████████████
A⁻B⁻	
A⁻B⁻ + A28	
A⁻B⁻ + B44	████████████
A⁻B⁻ + Cw7	

FIG. 4A

Stimulator Cells	TNF Released by CTL 159/5 (pg/ml)
	20 40 60 80
LB33-MEL.c1	███████████
COS	
COS + B44	
COS + B44 + cDNA 350/2	█████

FIG. 4B

Stimulator Cells	TNF Released by CTL 159/5 (pg/ml)
	20 40 60 80
JAR + B44	▌
JAR + B44 + cDNA 350/2	████

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FIG. 5A

FIG. 5B

FIG. 6

FIG. 7

FIG.8A

Stimulator Cells	TNF Released by CTL (pg/ml)
LB33-MEL.A-1	
A⁻B⁻D⁻	
A⁻B⁻D⁻ + A28	
A⁻B⁻D⁻ + B44	
A⁻B⁻D⁻ + Cw7	

FIG.8B

Stimulator Cells	TNF Released by CTL (pg/ml)
LB33-MEL.A-1	
A⁻D⁻	
A⁻D⁻ + A28	
A⁻D⁻ + B44	
A⁻D⁻ + Cw7	

FIG.8C

Stimulator Cells	TNF Released by CTL (pg/ml) 20 40 60 80
LB33-MEL.A-1	
C⁻	
C⁻ + A24	
C⁻ + B13	
C⁻ + Cw6	

FIG.8D

Stimulator Cells	TNF Released by CTL (pg/ml) 20 40 60 80
LB33-MEL.A-1	
C⁻	
C⁻ + A24	
C⁻ + B13	
C⁻ + Cw6	

FIG.8E

Stimulator Cells	TNF Released by CTL (pg/ml)				
	20	40	60	80	100
LB33-MEL.A-1	████████████████████████████				
A⁻B⁻D⁻	▯				
A⁻B⁻D⁻ + A28	▯				
A⁻B⁻D⁻ + B44	▯				
A⁻B⁻D⁻ + Cw7	▯				

FIG.8F

Stimulator Cells	TNF Released by CTL (pg/ml)				
	20	40	60	80	100
A⁻B⁻	▯				
A⁻B⁻ + A28	▯				
A⁻B⁻ + B44	▯				
A⁻B⁻ + Cw7	▯				

FIG. 9

FIG. 10A

FIG. 10B

FIG. 10C

Stimulator Cells	TNF Released by CTL 269/1 (pg/ml)			
	20	40	60	80
LB33-MEL.B-1				
LB33-MEL.B-1 +anti-A24				

FIG. 11B

● MEVDPIGHLY	MAGE-3.B44	
⊟ SEIWRDIDF	TYROSINASE.B44	
⊸ FLRGRAYGL	EBNA-3A.B8	

FIG. 11A

● MEVDPIGHLY	MAGE-3.B44	
⊟ SEIWRDIDF	TYROSINASE.B44	
⊸ FLRGRAYGL	EBNA-3A.B8	

FIG. 12B

FIG. 12A

FIG. 13A

FIG. 13B

FIG. 14B

FIG. 14A

FIG. 15A

Donor: LB816
Target: B*4402
○ no peptide
● + peptide

% Specific Lysis

Lymphocyte to target ratio

FIG. 15B

Donor: LB822
Target: B*4403
○ no peptide
● + peptide

% Specific Lysis

Lymphocyte to target ratio

FIG. 16B

FIG. 16A

FIG. 17

FIG. 18A

FIG. 18B

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C07K 7/06; C12N 5/06, 5/08; G01N 33/574

US CL :530/328, 240.1; 435/7.24

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/328, 240.1; 435/7.24

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, CA, MEDLINE, EMBASE, BIOSIS
search terms: peptide, HLA-B44, T cell, T lymphocyte

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A, P	US 5,558,995 A (VAN DER BRUGGEN ET AL.) 24 September 1996.	1-6
A, P	US 5,541,104 A (CHEN ET AL.) 30 July 1996.	1-6
A	US 5,342,774 A (BOON ET AL.) 30 August 1994.	1-6

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance		
"E"	earlier document published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
31 MARCH 1997	**11 APR 1997**

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	ANTHONY C. CAPUTA
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

FIG. 1A

FIG. 1B

FIG. 1C

FIG. 2

FIG. 3

Stimulator Cells	TNF Released by CTL 159/5 (pg/ml)
	20 40 60 80 100
LB33-MEL.c1	████████████████████████
A⁻B⁻	
A⁻B⁻ + A28	
A⁻B⁻ + B44	████████████████
A⁻B⁻ + Cw7	

FIG. 4A

Stimulator Cells	TNF Released by CTL 159/5 (pg/ml)
	20 40 60 80
LB33-MEL.c1	██████████████
COS	
COS + B44	
COS + B44 + cDNA 350/2	██████

FIG. 4B

Stimulator Cells	TNF Released by CTL 159/5 (pg/ml)
	20 40 60 80
JAR + B44	▏
JAR + B44 + cDNA 350/2	████

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FIG. 5A

FIG. 5B

FIG.8A

Stimulator Cells	TNF Released by CTL (pg/ml)
LB33-MEL.A-1	▓▓▓▓▓▓▓▓▓▓▓▓ (~102)
A⁻B⁻D⁻	▪ (~2)
A⁻B⁻D⁻ + A28	▪ (~2)
A⁻B⁻D⁻ + B44	▓▓▓▓▓▓▓ (~72)
A⁻B⁻D⁻ + Cw7	▪ (~2)

Scale: 20 40 60 80 100

FIG.8B

Stimulator Cells	TNF Released by CTL (pg/ml)
LB33-MEL.A-1	▓▓▓▓▓ (~28)
A⁻D⁻	▪ (~2)
A⁻D⁻ + A28	▓▓▓▓▓ (~29)
A⁻D⁻ + B44	▪ (~2)
A⁻D⁻ + Cw7	▪ (~2)

Scale: 20 40 60

FIG.8C

Stimulator Cells	TNF Released by CTL (pg/ml)			
	20	40	60	80
LB33-MEL.A-1				
C⁻				
C⁻ + A24				
C⁻ + B13				
C⁻ + Cw6				

FIG.8D

Stimulator Cells	TNF Released by CTL (pg/ml)			
	20	40	60	80
LB33-MEL.A-1				
C⁻				
C⁻ + A24				
C⁻ + B13				
C⁻ + Cw6				

FIG. 7

FIG. 6

FIG.8E

Stimulator Cells	TNF Released by CTL (pg/ml)
	20 40 60 80 100
LB33-MEL.A-1	▓▓▓▓▓▓▓▓▓▓▓▓▓▓
A⁻B⁻D⁻	▯
A⁻B⁻D⁻ + A28	▯
A⁻B⁻D⁻ + B44	▯
A⁻B⁻D⁻ + Cw7	▯

FIG.8F

Stimulator Cells	TNF Released by CTL (pg/ml)
	20 40 60 80 100
A⁻B⁻	▯
A⁻B⁻ + A28	▯
A⁻B⁻ + B44	▯
A⁻B⁻ + Cw7	▯

FIG. 9

FIG. 10A

(Y-axis: % Specific Lysis, 0 to 60; X-axis: Effector/ Target Ratio, 1, 3, 10, 30)

FIG. 10B

(Y-axis: % Specific Lysis, 0 to 60; X-axis: Effector/ Target Ratio, 1, 3, 10, 30)

FIG. 10C

Stimulator Cells	TNF Released by CTL 269/1 (pg/ml)			
	20	40	60	80
LB33-MEL.B-1				
LB33-MEL.B-1 +anti-A24				

BNSDOCID: <WO___9731017A1TI_>

FIG. 11B

FIG. 11A

FIG. 12B

FIG. 12A

FIG. 13A

FIG. 13B

FIG. 14A

FIG. 14B

FIG. 15A

FIG. 15B

FIG. 16B

FIG. 16A

FIG. 17

FIG. 18A

FIG. 18B